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Purification and Characterization of a Novel Nitrilase of *Rhodococcus rhodochrous* K22 That Acts on Aliphatic Nitriles

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A novel nitrilase that preferentially catalyzes the hydrolysis of aliphatic nitriles to the corresponding carboxylic acids and ammonia was found in the cells of a facultative crotononitrile-utilizing actinomycete isolated from soil. The strain was taxonomically studied and identified as *Rhodococcus rhodochrous*. The nitrilase was purified, with 9.08% overall recovery, through five steps from a cell extract of the stain. After the last step, the purified enzyme appeared to be homogeneous, as judged by polyacrylamide gel electrophoresis, analytical centrifugation, and double immunodiffusion in agarose. The relative molecular weight values for the native enzyme, estimated from the ultracentrifugal equilibrium and by high-performance liquid chromatography, were approximately 604,000 ± 30,000 and 650,000, respectively, and the enzyme consisted of 15 to 16 subunits identical in molecular weight (41,000). The enzyme acted on aliphatic olefinic nitriles such as crotononitrile and acrylonitrile as the most suitable substrates. The apparent K_m values for crotononitrile and acrylonitrile were 18.9 and 1.14 mM, respectively. The nitrilase also catalyzed the direct hydrolysis of saturated aliphatic nitriles, such as valeronitrile, 4-chlorobutyronitrile, and glutaronitrile, to the corresponding acids without the formation of amide intermediates. Hence, the *R. rhodochrous* K22 nitrilase is a new type distinct from all other nitrilases that act on aromatic and related nitriles.

Nitrile compounds are synthesized on a large scale as solvents, plastics, synthetic rubber, pharmaceuticals, herbicides, and starting materials from other industrially important chemicals. However, they are notoriously poisonous. Crotononitrile and acrylonitrile, which have been proposed as specific reagents for the alkylation of protein sulfhydryl groups (7, 14), are not only toxic to the central nervous system of animals but also mutagenic by virtue of their conjugated bonds (26, 47). Nitrile waste dispersed throughout the biosphere has been recognized as an important form of environmental pollution.

In recent years, the most significant development in the field of synthetic organic chemistry has been the application of biological systems to chemical reactions. Reactions catalyzed by cells and enzyme systems display far greater specificities than more conventional organic reactions.

The microbial degradation of nitriles has been found to proceed through two enzymatic pathways. Nitrilase catalyzes the direct cleavage of nitriles to yield the corresponding acids and ammonia, whereas nitrile hydratase catalyzes the hydration of nitriles to amides. Both nitrile-converting enzymes have attracted increasing attention as catalysts for processing organic chemicals, since they can convert nitriles to the corresponding higher-value acids or amides. Recently, the use of *Pseudomonas chlororaphis* B23 nitrile hydratase, which was found in our laboratory, for the industrial production (6,000 ton per year) of the important chemical commmodity acrylamide was pioneered in Japan (3, 36). We have purified and characterized the nitrile hydratases of *P. chlororaphis* B23 (30), *Brevibacterium* sp. strain R312 (31), and *Rhodococcus rhodochrous* J1 (32). On the other hand, we recently found an abundance of nitrilase in cells of *R. rhodochrous* J1 (29) and established the optimal conditions for the production of vitamins, nicotinic acid (24), and *p*-aminobenzoic acid (21) with resting cells. We have purified and characterized the nitrilase from *R. rhodochrous* J1 (20).

Nitrilases that utilize benzonitrile and related aromatic nitriles as substrates have been purified from *Pseudomonas* sp. (19, 35), *Nocardia* sp. strains NCIB 11215 (18) and NCIB 11216 (16), *Fusarium solani* (17), *Arthrobacter* sp. (4), *R. rhodochrous* J1 (20), and *Escherichia coli* transformed with a *Klebsiella ozaenae* plasmid DNA (38). However, aliphatic nitriles are inactive as substrates for all of these nitrilases. It has been shown that aliphatic nitriles are catabolized via amides by the combination of nitrile hydratase and amidase (2, 3, 13, 27, 47). To the best of our knowledge, no nitrilases that act on aliphatic nitriles have been reported. Such a nitrilase would be an interesting enzyme from the viewpoints of amelioration of the environmental pollution due to artificial nitriles, detoxification, and bioconversion. We have described the occurrence of a novel nitrilase that acts preferentially on aliphatic nitriles in *R. rhodochrous* K22 isolated from soil. In the work described here, the enzyme, tentatively designated as an aliphatic nitrilase, was purified and characterized.

MATERIALS AND METHODS

Materials. DEAE-Sephacel, phenyl-Sepharose CL-4B, and a low-molecular-weight standard kit were obtained from Pharmacia (Uppsala, Sweden). Cellulofine GCL-2000 superfine was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Marker proteins for molecular mass determination by high-performance liquid chromatography (HPLC) were purchased from Oriental Yeast Co. (Tokyo, Japan). All other chemicals used were from commercial sources and of reagent grade.

Screening and identification. *R. rhodochrous* K22 was isolated from soil samples by means of an enrichment culture technique. Crotononitrile-utilizing bacteria were isolated as follows. A soil sample (5 g) was added to a 500-ml shaking flask containing 80 ml of medium (pH 7.2) consisting of 0.5 g of glycerol, 2 g of K_2HPO_4, 1 g of NaCl, 0.2 g of

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$MgSO_4 \cdot 7H_2O$, 1 ml of a vitamin mixture (2 mg of nicotinic acid, 0.4 g of thiamine hydrochloride, 0.2 g of *p*-aminobenzoic acid, 0.2 g of riboflavin, 10 mg of folic acid, and 0.4 g of pyridoxime hydrochloride in 1 liter of distilled water) and crotononitrile at a final concentration of 0.01% (vol/vol)/liter of tap water. Cultivation was performed with shaking at 28°C for 1 week. Once a week, 0.5 volume (40 ml) of the isolation medium was replaced with 40 ml of fresh medium, and crotononitrile was fed to the medium at the concentration of 0.05% (vol/vol). After 1 month of cultivation, the microorganisms were spread on agar plates and isolated.

Isolation was carried out according to Goodfellow (15). The chemical compositions of cells were analyzed by the methods of Becker et al. (5), Lechevalier and Lechevalier (23), Suzuki and Komagata (42), Alshamaony et al. (1), and Uchida and Aida (44).

Culture conditions. *R. rhodochrous* K22 was collected from an agar slant and inoculated into the subculture medium. The subculture was carried out at 28°C for 30 h with reciprocal shaking in a test tube containing 4 ml of a medium consisting of 5 g of Polypepton (Daigo, Osaka, Japan), 5 g of meat extract (Mikuni, Tokyo, Japan), 0.5 g of yeast extract (Oriental Yeast Co.), and 2 g of NaCl per liter of tap water (pH 7.0). Then 8 ml of the subculture was inoculated into a 2-liter shaking flask containing 500 ml of a medium consisting of 50 g of sorbitol, 3 g of yeast extract, 7 g of NZ amine (Humko Sheffield Chemical), 7 g of urea, and 1 ml of isovaleronitrile per liter of tap water (pH 7.2). Incubation was carried out at 28°C with reciprocal shaking. At 76 and 100 h during the cultivation, 0.1 and 0.2% (vol/vol) isovaleronitrile were fed, respectively, followed by further incubation for 20 h. At 120 h from the start, the cells were harvested by continuous-flow centrifugation at 10,000 × *g* at 4°C and then washed twice with 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. The yield of wet cells was approximately 3.5 g/liter of medium.

Eight strains were used for studies of the immunological properties of the nitrilase. The microorganisms came from stock cultures of the Institute of Fermentation, Osaka (IFO strains), the Japan Collection of Microorganisms (JCM strains), and the American Type Culture Collection (ATCC strains). The following *Rhodococcus* strains were examined: *R. erythropolis* (IFO 12539 and IFO 12682), *R. rhodochrous* (IFO 3338, JCM 2157, JCM 3202, and ATCC 21198), *R. rhoseus* (JCM 2156), and *R. rubropertinctus* (JCM 3204). These *Rhodococcus* strains were cultivated for 48 h in medium consisting of 5 g of Polypepton, 3 g of malt extract (Difco Laboratories, Detroit, Mich.), 3 g of yeast extract, 10 g of glycerol, and 1 ml of isovaleronitrile per liter of tap water (pH 7.2). Cells harvested by centrifugation at 9,000 × *g* at 4°C for 20 min were washed with 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol and then disrupted for 50 min by ultrasonic oscillation (19 kHz; Insonator model 201M; Kubota, Tokyo, Japan). The cell debris was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant solution was designated as the cell extract.

Preparation of resting cells. Cells were harvested from culture both by centrifugation at 10,000 × *g* for 20 min. The harvested cells were washed with 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol and then suspended in the same buffer. This cell suspension was used for the resting-cell reaction.

Enzyme assay and definition of units. Nitrilase activity was assayed in a reaction mixture (2 ml) consisting of 20 μmol of potassium phosphate buffer (pH 8.0), 200 μmol of crotononitrile, 1 μmol of dithiothreitol, and an appropriate amount of cells or enzyme. The reaction was carried out at 25°C for 10 min and stopped by adding 0.2 ml of 1 M HCl to the reaction mixture. The amounts of crotonic acid and crotonamide formed in the reaction mixture were determined by HPLC with a Shimadzu LC-6A system equipped with an M&S pack C_{18} column (reverse-phase column; 4.6 by 150 mm; M&S Instruments, Osaka, Japan) at a flow rate of 1.0 ml/min, using the following solvent system: 5 mM KH_2PO_4-H_3PO_4 buffer (pH 2.9)–acetonitrile, 3:1 (vol/vol). One unit of enzyme is defined as the amount needed to catalyze the formation of 1 μmol of crotonic acid per min from crotononitrile under the conditions described above.

Protein was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford (6), using dye reagent supplied by Bio-Rad Laboratories (Richmond, Calif.). For the nitrilase from *R. rhodochrous* K22, protein was determined from the A_{280}. The absorption coefficient was calculated to be 1.09 ml/mg per cm by absorbance and dry-mass determinations. Spectrophotometric measurements were performed with a Shimadzu UV-240 spectrophotometer. Specific activity is expressed as units per milligram of protein.

Substrate specificity. The standard reaction mixture (2 ml) was composed of 0.2 mmol of potassium phosphate buffer (pH 7.5), 0.2 mmol of nitrile, 1 μmol of dithiothreitol, 0.2 ml of methanol, and an appropriate amount of the enzyme. Methanol was added to enhance the solubility of the substrate, the enzyme being stable even in the presence of 10% (vol/vol) methanol (data not shown). However, in the cases of aromatic nitriles, heterocyclic nitriles, alicyclic nitriles, and aralkyl nitriles, the reaction was carried out with a final concentration of 10 mM because of their low solubility in water. Each reaction was carried out at 25°C for 10 to 50 min and stopped by adding 0.2 ml of 1 M HCl to the reaction mixture. The amount of NH_3 produced in the reaction mixture was colorimetrically estimated by the phenol-hypochlorite method (12), using a Conway microdiffusion apparatus (10).

The following nitrile compounds were tested for substrate specificity: the aliphatic olefinic nitriles crotononitrile, acrylonitrile, methacrylonitrile, 3-ethoxyacrylonitrile, 3-aminocrotononitrile, 2-chloroacrylonitrile, fumaronitrile, diaminomaleonitrile, 2-methyl-2-butenenitrile, 2-methyl-3-butenenitrile, 2-pentenenitrile, 3-pentenenitrile, 1,4-dicyano-2-butene, 2,4-dicyano-1-butene, and *N*-cyano-*N'*-*S*-dimethylisothiourea; the saturated aliphatic nitriles acetonitrile, propionitrile, butyronitrile, isobutyronitrile, valeronitrile, isovaleronitrile, capronitrile, isocapronitrile, trimethylacetonitrile, methoxyacetonitrile, (*S*)-(+)-2-methylbutyronitrile, chloroacetonitrile, (±)-2-chloropropionitrile, 3-chloropropionitrile, 4-chlorobutyronitrile, cyanoacetic acid, cyanoacetic acid ethyl ester, 2-cyanoacetamide, 3-cyano-L-alanine, *N*-methyl-β-alaninenitrile, lactonitrile, maleonitrile, succinonitrile, glutaronitrile, adiponitrile, pimelonitrile, suberonitrile, sebaconitrile, 3,3'-oxydipropionitrile, iminodiacetonitrile, and 3,3'-iminodipropionitrile; the aromatic nitriles benzonitrile, monosubstituted (position 2, 3, or 4) benzonitrile derivatives with a functional group (e.g., a hydroxy, amino, methyl, nitro, cyano, methoxy, ethoxy, or carboxyl group), 4-cyanothiophenol, 1-or 2-cyanonaphthalene, 9-cyanoanthracene, 4-biphenylcarbonitrile, 4,4'-biphenyldicarbonitrile, 2,6-dichlorobenzonitrile, and 3,5-diiodo-4-hydroxybenzonitrile; the heteroaromatic nitriles 2-, 3-, or 4-cyanopyridine, cyanopyrazine, 2,3-dicyanopyrazine, 2-furonitrile, 2-thiophenecarbonitrile, 5-cyanoindole, piperonylonitrile, 3-quinoline-

carbonitrile, and 6-cyanopurine; the alicyclic nitriles cyclopropanecarbonitrile, 1,2-dicyanocyclobutane, cyclopentanecarbonitrile, cycloheptanecarbonitrile, 4-cyano-1-cyclohexene, 5-norbornene-2-carbonitrile, 1-adamantanecarbonitrile, 1-cyclopenteneacetonitrile, and 1-cyclohexeneacetonitrile; the aralkyl nitriles cinnamonitrile, phenylacetonitrile, (±)-α-aminophenylacetonitrile, 3-xylylene dicyanide, 3-indoleacetonitrile, 2-pyridineacetonitrile, 2-or 3-thiopheneacetonitrile, 2-furanacrylonitrile, α-methylphenylacetonitrile, and mandelonitrile; and the *N*-carbonitriles 1,4-piperazinedicarbonitrile, 4-morpholinecarbonitrile, 1-pyrrolidine 1carbonitrile, and 1-piperidinecarbonitrile.

Purification of *R. rhodochrous* K22 nitrilase. All purification steps were performed at 0 to 4°C. Throughout purification steps 1 to 5, potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol was used unless otherwise specified. Centrifugation was carried out for 30 min at 12,000 × *g*.

(i) Step 1. Preparation of a cell extract. Washed cells (35 g) from 20 liters of culture broth were suspended in 1.0 liter of 0.1 M buffer and then disrupted with a Dyno-Mill (W. A. Bachofen, Basel, Switzerland), followed by sonication at 19 kHz for 20 min with an ultrasonic oscillator (model 4280; Kaijo Denki, Tokyo, Japan). The cell debris was removed by centrifugation. The resulting supernatant solution was used as the cell extract and followed by dialysis against 10 mM buffer.

(ii) Step 2. DEAE-Sephacel column chromatography. The dialyzed solution was applied to a DEAE-Sephacel column (5.2 by 50.5 cm) equilibrated with 10 mM buffer. After the column was washed thoroughly with 10 mM buffer followed by the same buffer containing 0.1 M KCl and 0.2 M KCl, the enzyme was eluted with 2.5 liters of 10 mM buffer containing 0.3 M KCl. The active fractions were pooled, and ammonium sulfate was added to give 80% saturation. After centrifugation of the suspension, the precipitate was dissolved in 0.1 M buffer, followed by dialysis against 10 mM buffer.

(iii) Step 3. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the resulting supernatant solution to give 40% saturation. The pH was maintained at 7.5 with an ammonia solution. After being stirred for 4 h or more, the precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant solution to give 50% saturation. The suspension was then centrifuged, and the pellet was dissolved in 0.1 M buffer, followed by dialysis for 36 h against four changes of 10 liters of 10 mM buffer.

(iv) Step 4. Phenyl-Sepharose CL-4B column chromatography. After the enzyme solution from step 3 was cooled, sodium chloride was added in small portions with stirring to bring the solution to 1.6 M saturation. The enzyme solution was placed on a phenyl-Sepharose CL-4B column (2.5 by 27.5 cm) equilibrated with 20 mM buffer containing 1.6 M saturated sodium chloride. After the column was washed thoroughly with 20 mM buffer containing 1.6 M sodium chloride, the enzyme was eluted by lowering the ionic strength of sodium chloride (1.6 to 1.0 M) in the same buffer. The active fractions were combined and precipitated with ammonium sulfate at 80% saturation.

(v) Step 5. Cellulofine GCL-2000 superfine column chromatography. The precipitate from step 4 was centrifuged and then dissolved in 0.1 M buffer. This enzyme solution was placed on a Cellulofine GCL-2000 superfine column (1.8 by 101 cm) equilibrated with 10 mM buffer. The rate of sample loading and column elution was maintained at 5 ml/h with a peristaltic pump (LKB 2121). The active fractions were combined and then precipitated with ammonium sulfate at a final concentration of 80%. The precipitate was centrifuged and then dissolved in 0.1 M buffer. The enzyme solution was dialyzed against 10 mM buffer and then preserved in 10 mM buffer containing 50% (vol/vol) glycerol at −20°C.

Analytical methods for the nitrilase. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in 12.5% polyacrylamide slab gels, using the Tris-glycine buffer system (22). Proteins were stained with Coomassie brilliant blue R-250 and destained in ethanol-acetic acid-H_2O (3:1:6, vol/vol/vol). The relative molecular weight of the subunit of the enzyme was determined from the relative mobilities of standard proteins.

To estimate the molecular weight of the enzyme, the enzyme sample (20 μg) was subjected to HPLC (Toyo Soda CO-8000 system) on a TSK G-4000SW column (0.75 by 60 cm; Toyo Soda) at a flow rate of 0.5 ml/min, using 0.1 M *N*-2-hydroxyethyl-*N'*-2-ethanesulfonic acid (HEPES)-KOH buffer (pH 7.0) containing 0.2 M NaCl at room temperature. The A_{280} of the effluent was recorded. The relative molecular weight of the enzyme was then calculated from the relative mobility compared with those of the standard proteins, glutamate dehydrogenase (M_r = 290,000), lactate dehydrogenase (140,000), enolase (67,000), adenylate kinase (32,000), cytochrome *c* (12,400) (products of Oriental Yeast Co.), thyroglobulin (bovine; M_r = 669,000; Sigma Chemical Co., St. Louis, Mo.), alcohol oxidase (*Candida boidinii*; M_r = 673,000; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and β-galactosidase (type II; *E. coli*; M_r = 540,000; Toyobo Co., Osaka, Japan).

Analytical ultracentrifugation was carried out with a Spinco model E ultracentrifuge at 20°C. The purity of the enzyme and its sedimentation coefficient were determined with a phase plate as a Schlieren diagram and operated at 12,590 rpm, with the boundary at the meniscus in the sector-shaped centrifuge cell (43). The relative molecular weight was determined by the procedure of Van Holde and Baldwin (46), using Rayleigh interference optics. Multicell operations were carried out for three samples of different initial concentrations, ranging from 1.40 to 5.62 mg/ml, using an An-G rotor and double cells with different side-wedge angles. The rotor was centrifuged at 3,848 rpm at 15°C for 16 h, and the interference pattern was photographed at 30-min intervals. The relationship between the concentration of the enzyme and the fringe shift was determined by using a synthetic-boundary cell.

Amino acid composition. For analysis of the amino acid composition of the nitrilase, duplicate samples of the enzyme were hydrolyzed in 6 M HCl in evacuated and sealed Pyrex tubes at 110°C for 20, 42, and 70 h. The hydrolysates were analyzed with an amino acid analyzer (K101-AS; Kyowa Seimitsu, Mitaka, Japan) by the procedure of Spackman et al. (37). Cysteine and cystine were determined as cysteic acid after performic acid oxidation of the sample by the method of Moore (28). Tryptophan was determined spectrophotometrically at 280 and 288 nm in the presence of 6 M guanidine hydrochloride by the method of Edelhoch (11).

Metal analysis. All glassware was boiled briefly in 2 M HCl and then exhaustively rinsed with distilled water before use. Before analysis, the enzyme was dialyzed against 10 mM potassium phosphate buffer (pH 7.5). Enzyme samples containing 1.0 to 3.0 mg of protein per ml were analyzed with an inductively coupled radiofrequency plasma spectrophotometer (Shimadzu ICPV-1000; 27,120 MHz). The following assay conditions were used for qualitative analysis: cooling gas, 15 liters/min; plasma gas, 1.2 liters/min; and carrier gas,

1.0 liter/min. The spectra were scanned, from 400 nm to 190 nm, at a speed of 25 nm/min.

Antiserum preparation. Antibodies were elicited by injection of 8.0 mg of *R. rhodochrous* K22 nitrilase homogenized with an equal volume of complete Freund adjuvant (Difco) into young white male rabbits. The rabbits received a booster injection after 3 weeks. The booster injection, administered subcutaneously in the neck, was of 3.5 mg of antigen homogenized in an equal volume of incomplete Freund adjuvant (Difco). On the day 7 after the booster injection, blood was collected and serum was prepared. Ouchterlony plates were made using 1% (wt/vol) special Noble agar (Difco) in 10 mM Tris-H_2SO_4 (pH 8.0) containing 0.01% sodium azide (34).

RESULTS AND DISCUSSION

Identification of the microorganism. We isolated from a soil sample a strain, K22, that utilizes crotononitrile as a sole nitrogen source. Taxonomical studies on strain K22 were carried out. The microbiological characteristics of strain K22 were as follows. Morphologically, the cells were gram positive, aerobic, non-endospore forming, non-acid fast, and nonmotile. Cocci germinated and gave rise to branched filaments, which underwent fragmentation into rods (0.5 to 1.1 μm by 1.5 to 5.0 μm). Elongated cells and snapping division were frequently observed. Physiological characteristics were as follows: reduction of nitrate, positive; denitrification, methyl red test, indole production, hydrogen sulfide production, and Voges-Proskauer test, negative; oxidase, negative; catalase and phosphatase, positive; growth range, pH 5 to 10; growth temperature, 10 to 41°C; growth in 7% NaCl, positive; oxygen requirement, aerobic; Hugh-Leifson test, weakly oxidative; acid production from glucose, positive; gas production from glucose, negative; decomposition of tyrosine, positive; decomposition of adenine and urea, negative; growth on sole carbon sources, positive with 1% (wt/vol) maltose, mannitol, sorbitol, and trehalose and with 0.1% *m*-and *p*-hydroxybenzoic acid, sodium adipate, sodium benzoate, sodium citrate, sodium lactate, testosterone, acetamide, and sebacic acid and negative with 1% inositol and rhamnose; hydrolysis of starch, negative; Tween 80 hydrolysis, positive; *o*-nitrophenyl-β-D-galactopyranoside hydrolysis, negative. The chemical composition of the cells was as follows: *meso*-diaminopimelic acid, arabinose, and galactose, present; major fatty acid components, hexadecanoic acid (31.4%), hexadecenoic acid (15.0%), octadecenoic acid, (15.4%), and methyloctadecanoic acid (tuberculostearic acid) (19.2%); C_{32} to C_{46} mycolic acids of the mycolic acid type, present. The strain was positive for the glycolate test, and its G+C content of DNA was 67 to 70% mol (T_m).

Thus, strain K22 is pleomorphic and has some typical characteristics of a coryneform bacterium. Recently, chemical examination of the cell wall provided data useful for distinguishing coryneform bacteria at the generic level. From its morphology and physiology and from the presence of *meso*-diaminopimelic acid, arabinose, and galactose (23), fatty acids of the straight-chain saturated and monounsaturated types, and 10-methyl branched-chain acids (42), strain K22 was thought to belong to the genus *Rhodococcus*, *Corynebacterium*, or *Nocardia*. To clearly differentiate between *Corynebacterium* and related taxa, the types of mycolic acids and the acyl type in the cell wall were determined (8, 45). Strain K22 was found to possess mycolic acids with 32 to 46 carbon atoms and glycolyl residues of the glycan moiety. From these results, it was assumed that strain K22 belongs to the genus *Rhodococcus*. Then, in accordance with references 15 and 39, strain K22 was classified as *Rhodococcus rhodochrous*.

TABLE 1. Purification of the aliphatic nitrilase from *R. rhodochrous* K22

Step	Total protein[a] (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
1. Cell-free extract	6,110	544	0.0890	100
2. DEAE-Sephacel	1,980	201	0.102	36.9
3. $(NH_4)_2SO_4$ (0.4–0.5)	731	102	0.140	18.8
4. Phenyl-Sepharose CL-4B	160	53.4	0.334	9.81
5. Cellulofine GCL-2000 superfine	67.0	49.4	0.737	9.08

[a] The concentration of the purified enzyme after step 5 was determined from the A_{280}, using the extinction coefficient ($A^{1\%}$ = 10.9).

During the screening program for nitrilases, among a number of nitrile-degrading microorganisms, only a very few nitrilase-containing microorganisms were found in the environment, whereas nitrile hydratase-containing organisms were found to be widespread (data not shown). Thus, the occurrence of an aliphatic nitrilase is rare even among nitrilase-containing microorganisms.

Purification of the aliphatic nitrilase. By using the purification procedures described in Materials and Methods, the enzyme was purified 8.3-fold with a yield of 9.08% from the cell extract, using crotononitrile as the substrate (Table 1). The purified enzyme showed only one band on SDS-polyacrylamide slab gel electrophoresis (Fig. 1, lane B). It sedimented as a single symmetrical peak in 10 mM potassium phosphate buffer (pH 7.5) on analytical ultracentrifugation. Further evidence for the purity of the enzyme preparation was provided by the results of HPLC on a TSK

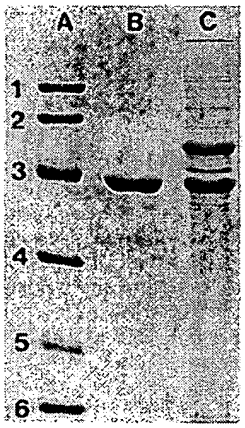

FIG. 1. SDS-polyacrylamide gel electrophoresis of the aliphatic nitrilase. Conditions are given in Materials and Methods. Lane A was loaded with the following molecular weight standards: 1, phosphorylase *b* (94,000); 2, bovine serum albumin (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000); 5, soybean trypsin inhibitor (20,100); and 6, α-lactalbumin (14,400). Lane B was loaded with the purified aliphatic nitrilase (27 μg). Lane C was loaded with 82 μg of protein of a cell extract of cells cultivated for 60 h in medium consisting of 50 g of sorbitol, 3 g of yeast extract, 7 g of NZ amine, 7 g of urea, and 1 ml of isovaleronitrile per liter of tap water (pH 7.2), with feeding of 0.2% (vol/vol) isovaleronitrile at 48 h from the start. The direction is from top (cathode) to bottom (anode).

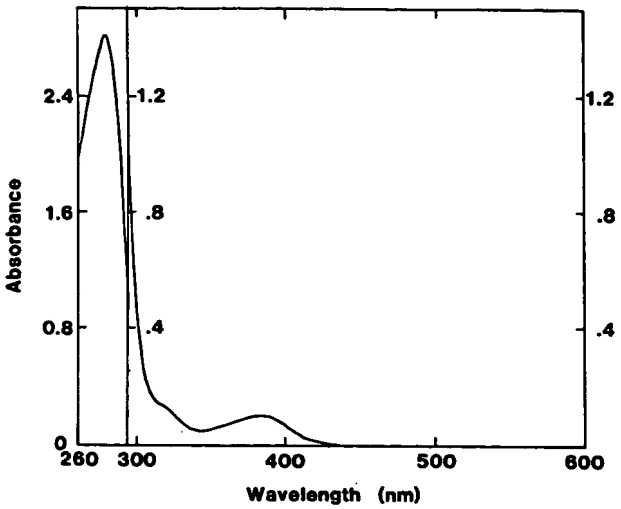

FIG. 2. Absorption spectrum of the native aliphatic nitrilase. The concentration of enzyme protein was 2.57 mg/ml in 0.01 M potassium phosphate buffer (pH 7.5). The same buffer was examined as a control.

G-4000SW column, which showed a symmetrical single protein peak. The purity of the purified enzyme was also examined by double immunodiffusion in agarose. A single precipitin line was observed between antibodies and the crude cell extract of *R. rhodochrous* K22. The purified enzyme catalyzed the hydrolysis of crotononitrile to crotonic acid at 0.737 μmol/min per mg of protein under the standard reaction conditions.

Molecular weight and subunit structure. The sedimentation coefficient ($s_{20,w}$) and diffusion coefficient ($D_{20,w}$) of the enzyme were calculated to be 10.9S (20°C; 10 mM potassium phosphate buffer, pH 7.5) and 6.27×10^{-6} cm²/s, respectively. A relative molecular weight of 604,000 ± 30,000 was determined by the sedimentation equilibrium method (46), assuming a partial specific volume of 0.74. The molecular weight of the enzyme was determined to be 650,000 by analytical HPLC. When the enzyme was treated with 1% SDS and 50 mM 2-mercaptoethanol and then electrophoresed on a gel containing 0.1% SDS, a single band was observed upon protein dyeing (Fig. 1, lane B). The molecular weight corresponding to the band was estimated to be 41,000, as judged from its mobility relative to those of reference proteins. Thus, the enzyme probably consists of 15 to 16 subunits identical in molecular weight; this molecular weight is closer to those of the enzymes found in *Nocardia* (M_r = 560,000; 16, 18) and *Fusarium* (M_r = 620,000; 17) species.

The purified enzyme showed maximum absorption at 278 nm and weak absorption at 320 and 380 nm (Fig. 2); such phenomena have not been reported for any other nitrilases. Even when purification of the enzyme was carried out three times in the same manner, the enzyme showed this absorption pattern, which was not eliminated upon thorough dialysis against 10 mM potassium phosphate buffer (pH 7.5). These findings suggest that a cofactor may bind to the enzyme. However, when crotononitrile, *p*-chloromercuribenzoate, $HgCl_2$, or H_2O_2 was added at a final concentration of 0.1 M, 10 mM, 5 mM, or 5 mM, respectively, to 7 nmol of the enzyme in 1.0 ml of 10 mM potassium phosphate buffer (pH 7.5), no appreciable spectral shift occurred.

TABLE 2. Amino acid composition of the aliphatic nitrilase[a]

Amino acid	No. of residues/subunit	
	Found	Integral
Aspartic acid[b]	41.0	41
Threonine[c]	16.9	17
Serine[c]	18.5	19
Glutamic acid[b]	38.0	38
Proline	20.0	20
Glycine	26.3	26
Alanine	43.2	43
Valine	19.7	20
Methionine	5.80	6
Isoleucine	18.3	18
Leucine	33.6	34
Tyrosine	15.5	16
Phenylalanine	11.4	11
Lysine	19.2	19
Histidine	10.1	10
Arginine	21.1	21
Tryptophan	5.82	6
Half-cystine	0.75	1

[a] Except for those amino acids determined independently and those estimated by extrapolation, the results are within ±5%. There was no significant difference in amino acid residues between the 20-h hydrolysate and the 42-or 70-h hydrolysate; each value is the average of the values for these hydrolysates.
[b] Both free and amide residues.
[c] Determined by extrapolation to time zero, assuming first-order decay.

Further experiments are in progress to identify the substances showing absorbance at 320 and 380 nm to elucidate the nitrilase reaction mechanism. Qualitative analysis of the following metals in the concentrated enzyme solution was performed with an inductively coupled radiofrequency plasma spectrophotometer: Be, B, Mg, Al, Si, P, S, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Sr, Zr, Mo, Pd, Ag, Cd, Sn, Sb, Ba, Ta, W, Pt, Au, Hg, Pb, La, and Ce. However, none of these 35 metals were detected within the limits of the assay (10 ng/ml). On the other hand, the nitrile hydratases of *Pseudomonas* (30) and *Brevibacterium* (31) species contain tightly bound iron (40, 41), and the *R. rhodochrous* J1 nitrile hydratase contains cobalt (32). In this respect, strain K22 nitrilase is quite different from these nitrile hydratases.

Amino acid composition. The enzyme contained all of the common amino acids (Table 2). The amino acid composition of our enzyme resembles those of the *R. rhodochrous* J1 nitrilase (20) and the *Klebsiella* enzyme (38) (obtained from the nucleotide sequence) except for valine, threonine, and lysine and for aspartic acid, leucine, and lysine, respectively. The minimum value with the nearest integral numbers of all amino acids found was calculated to be 40,671, which coincides well with the subunit molecular weight (41,000) determined by SDS-polyacrylamide gel electrophoresis.

Stability. The highly purified enzyme could be stored for more than 2 months at 4°C and for 11 months or more at −20°C without loss of activity in a 50% glycerol solution containing 10 mM potassium phosphate buffer (pH 7.5) and 1 mM dithiothreitol.

The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 1 h in 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, a sample of the enzyme solution was taken and the nitrilase activity was assayed under the standard conditions. It exhibited the following activity: 55°C, 0%; 50°C, 6.7%; 45°C, 28%; 40°C, 87%; 35°C, 92%; 30°C, 94%; and 25°C, 100%.

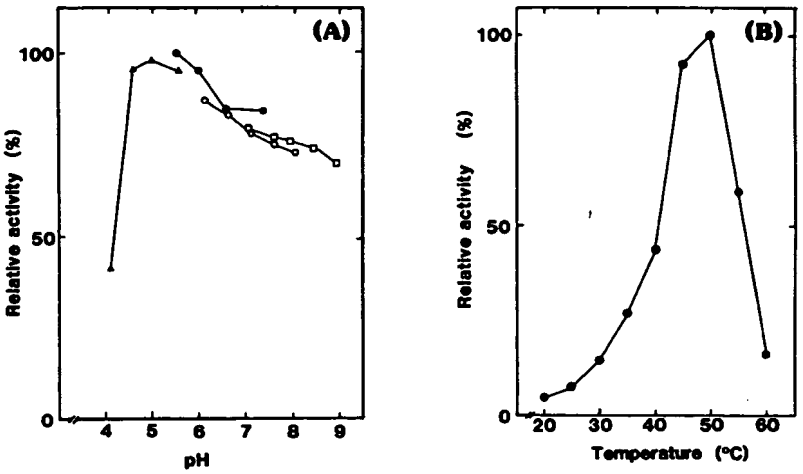

FIG. 3. Effects of pH (A) and temperature (B) on activity of the aliphatic nitrilase. (A) The reactions were carried out for 20 min at 25°C in the following buffers (final concentration, 0.1 M): CH_3COONa-CH_3COOH (△), potassium phosphate (○), MES-NaOH (●), and Tris hydrochloride (□). (B) The reactions were carried out for 20 min at various temperatures. Relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions.

The stability of the enzyme was examined at various pHs. The enzyme was incubated at 25°C for 45 min in the following buffers (final concentration, 0.1 M): 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH (pH 5.5 to 7.0), potassium phosphate (pH 6.0 to 8.0), Tris hydrochloride (pH 7.5 to 9.0), and NH_4Cl-NH_4OH (pH 9.0 to 10.0). Then a sample of the enzyme solution was taken, and the nitrilase activity was assayed under the standard conditions. The enzyme was most stable in the pH range of 6.0 to 8.0, 40% of its initial activity being retained even at pH 10.0.

Effects of pH and temperature. The effect of pH on the activity of the enzyme was examined with crotononitrile as the substrate (Fig. 3A). This enzyme showed maximum activity in the more acidic region of pH 5.5, whereas the optimum pHs of all other nitrilases previously reported are in the range of 7.5 to 9 (4, 16–20, 35, 38). However, this nitrilase was similar to those of *Nocardia* sp. strain NCIB 11215 and *Fusarium* sp. with respect to the broad pH range. The optimal temperature was found to be 50°C. Above 55°C, enzyme activity was rapidly lost (Fig. 3B).

Inhibitors. Various compounds were investigated for their inhibitory effects on enzyme activity. Incubation was carried out at 25°C for 10 min in the standard reaction mixture containing a tested compound at 1 mM. The enzyme was strongly inhibited by $HgCl_2$ and H_2O_2 (0 and 3.7%, respectively, of the original activity). However, the enzyme showed relatively high resistance to the thiol reagents 5,5′-dithio-bis(2-nitrobenzoate), iodoacetate, and *N*-ethylmaleimide. At a concentration of 5 or 10 mM, *p*-chloromercuribenzoate caused appreciable inhibition. Carbonyl reagents such as hydroxylamine, phenylhydrazine, semicarbazide, cysteamine, aminoguanidine, L-or D-penicillamine, and D-cycloserine were not inhibitory toward the enzyme. This nitrilase seems to differ in this respect from the nitrile hydratase from *P. chlororaphis* B23, which contains pyrroloquinoline quinone as a prosthetic group (33). Chelating reagents, e.g., *o*-phenanthroline, 8-hydroxyquinoline, EDTA, α,α′-dipyridyl, and diethyldithiocarbamate, also had no significant effect on the enzyme. These results are in good agreement with the finding that this enzyme contains no metals.

The effects of reductants on the inhibition by thiol reagents and H_2O_2 were examined. After the enzyme, which was fully dialyzed against 10 mM potassium phosphate buffer (pH 7.5), had been incubated with each inhibitor (final concentration, 1 mM) for 30 min, the reaction was carried out in the standard reaction mixture without the addition of dithiothreitol. Each reductant was added to the reaction mixture described above, after the aforementioned procedure, and then the enzyme was assayed. The inhibition by $HgCl_2$ could be partially reversed by the addition of dithiothreitol, 2-mercaptoethanol, and reduced glutathione (46, 48, and 14%, recovery, respectively, compared with the activity without treatment with inhibitor [100%]). The inhibition by H_2O_2 could be partially reversed by the addition of dithiothreitol, 2-mercaptoethanol, and reduced glutathione (34, 19, and 29% recovery, respectively). All other nitrilases characterized so far are inactivated by thiol reagents and are therefore classified as sulfhydryl enzymes. The *R. rhodochrous* K22 enzyme was not inhibited by several thiol reagents that were inhibitory for the other nitrilases (4, 16–20, 35, 38). With respect to the active sulfhydryl group, this nitrilase seems to differ from other nitrilases.

Substrate specificity. The ability of the enzyme to catalyze the hydrolysis of various nitriles was examined (Table 3). The *R. rhodochrous* K22 enzyme is characteristic in its broad substrate specificity for aliphatic nitriles, which cannot be hydrolyzed by any nitrilases (4, 16–20, 35, 38). Aliphatic olefinic nitriles, especially low-molecular-mass ones with two to five carbon atoms, were remarkably active as substrates for the enzyme. Acrylonitrile is known to be an effective nucleophilic agent for the modification of protein sulfhydryl groups (7, 14). Acrylonitrile, despite its alkylating ability, was 3.5 times more suitable as a substrate than crotononitrile. When the Michaelis constants for acrylonitrile and crotononitrile were estimated from the Lineweaver-Burk plot, the former (K_m = 1.14 mM) was found to exhibit higher affinity for the enzyme than did the latter (K_m = 18.9 mM). Saturated aliphatic nitriles, e.g., acetonitrile, 4-chlorobutyronitrile, and glutaronitrile, were also attacked by the enzyme. Branched-chain saturated aliphatic nitriles with two to five carbon atoms, e.g., trimethylacetonitrile, lactonitrile, (*S*)-(+)-2-methylbutyronitrile, isovaleronitrile, and 3-cyano-L-alanine, were less effective substrates than the corre-

TABLE 3. Substrate specificity of the aliphatic nitrilase

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Crotononitrile	100[a]	Glutaronitrile	345
Acrylonitrile	348	Adiponitrile	110
Methacrylonitrile	143	Pimelonitrile	27.3
3-Ethoxyacrylonitrile	19.1	Suberonitrile	21.4
3-Aminocrotononitrile	30.3	Sebaconitrile	15.7
2-Chloroacrylonitrile	47.1	3,3'-Oxydipropionitrile	29.0
Fumaronitrile	27.4	Iminodiacetonitrile	261
Diaminomaleonitrile	18.6	3,3'-Iminodipropionitrile	19.6
2-Methyl-2-butenenitrile	14.9	Benzonitrile[b]	27.1
2-Methyl-3-butenenitrile	46.8	3-Tolunitrile[b]	67.9
2-Pentenenitrile	6.33	3-Nitrobenzonitrile[b]	74.5
3-Pentenenitrile	132	Isophthalonitrile[b]	66.1
1,4-Dicyano-2-butene	42.6	3-Methoxybenzonitrile[b]	24.8
2,4-Dicyano-1-butene	127	3-Ethoxybenzonitrile[b]	21.1
Acetonitrile	28.3	3-Cyanopyridine[b]	7.27
Propionitrile	12.7	4-Cyanopyridine[b]	9.71
Butyronitrile	18.0	Cyanopyrazine[b]	17.3
Isobutyronitrile	6.74	2-Furonitrile[b]	52.0
Valeronitrile	40.8	2-Thiophenecarbonitrile[b]	61.1
Capronitrile	38.7	Piperonylonitrile[b]	6.80
Isocapronitrile	41.5	Cyclopropanecarbonitrile[b]	22.9
Methoxyacetonitrile	37.4	Cyclopentanecarbonitrile[b]	11.7
Chloroacetonitrile	61.7	4-Cyano-1-cyclohexene[b]	7.03
(±)-2-Chloropropionitrile	6.80	1-Cyclopenteneacetonitrile[b]	24.9
3-Chloropropionitrile	113	1-Cyclohexeneacetonitrile[b]	18.2
4-Chlorobutyronitrile	116	Cinnamonitrile[b]	31.2
Cyanoacetic acid ethyl ester	117	Phenylacetonitrile[b]	27.3
N-Methyl-β-alaninenitrile	11.4	3-Xylylene dicyanide[b]	14.6
Malononitrile	45.1	2-Thiopheneacetonitrile[b]	73.5
Succinonitrile	271	3-Thiopheneacetonitrile[b]	66.3

[a] The synthesis of crotonic acid, corresponding to 0.737 μmol/min per mg of protein, was taken as 100%.
[b] Incubated at a final concentration of 10 mM.

sponding straight-chain ones such as propionitrile and butyronitrile. Steric hindrance of the enzyme by the side chain of the branched-chain nitriles clearly influences the rate of hydrolysis. As for aliphatic dinitriles, nitriles with three atoms between the two cyano groups, such as glutaronitrile and iminodiacetonitrile, were better substrates than those with four atoms, such as adiponitrile, 3,3'-oxydipropionitrile, and 3,3'-iminodipropionitrile. Furthermore, the hydrolysis rate became lower with an increase in the number of carbon atoms of the dinitriles. Nitriles with an aromatic or heterocyclic ring were hydrolyzed at much lower rates than the aliphatic nitriles mentioned above (compared with the activity toward acrylonitrile or glutaronitrile, the activity toward benzonitrile was only 7.8%). Compared with the activity toward crotononitrile (100%), there was slight formation of ammonia (1 to 5%) from the following nitriles: isovaleronitrile, (*S*)-(+)-2-methylbutyronitrile, cyanoacetic acid, 3-cyano-L-alanine, lactonitrile, 3-aminobenzonitrile, 4-tolunitrile, 4-nitrobenzonitrile, 4-methoxybenzonitrile, 4-ethoxybenzonitrile, 4,4'-biphenyldicarbonitrile, 3,5-diiodo-4-hydroxybenzonitrile, 2-cyanopyridine, 1,2-dicyanocyclobutane, 5-norbornene-2-carbonitrile, 3-indoleacetonitrile, 2-pyridineacetonitrile, α-methylphenylacetonitrile, 2-furanacrylonitrile, mandelonitrile, and 1,4-piperazinedicarbonitrile. Other nitriles (see Materials and Methods) were nearly inactive as substrates for our enzyme.

It has been previously shown that nitriles of which the cyano group is conjugated with a double bond, such as benzonitrile, can be directly hydrolyzed to the corresponding acids and ammonia by nitrilase (4, 16–20, 35, 38), whereas aliphatic nitriles are catabolized in two stages, via conversion to the corresponding amides and then to the acids and ammonia by nitrile hydratase and amidase, respectively (2, 13, 27, 30, 31). In the former respect, the *R. rhodochrous* K22 enzyme seems to exhibit similarity to known benzonitrile-specific nitrilases. Surprisingly, however, the *R. rhodochrous* K22 nitrilase showed significant activity toward saturated aliphatic nitriles. These findings are not in agreement with the well-known hypothesis mentioned above and lead to the conclusion that this enzyme is a novel type of nitrilase.

Specific and molecular activities. The specific activity of the aliphatic nitrilase was 0.737 μmol/min per mg of protein at 25°C under the standard assay conditions. Assuming the molecular weight of this enzyme to be 650,000, its molecular activity was calculated to be 0.479 kmol/min per mol of nitrilase at 25°C under the standard assay conditions. On the other hand, the molecular activities of the nitrile hydratases of *Pseudomonas* (30) and *Brevibacterium* (31) species were 184 and 161 kmol/min per mol of nitrile hydratase, respectively, at 20°C with propionitrile as the substrate. The marked difference in molecular activity between the nitrilase and nitrile hydratases might reflect differences with respect to reaction mechanisms and cofactors (33, 40, 41).

Stoichiometry. The stoichiometry of nitrile consumption and acid formation during the hydrolysis of nitriles was examined in a reaction mixture consisting of 20 μmol of potassium phosphate buffer (pH 8.0), 1 μmol of dithiothreitol, 0.2 mmol of crotononitrile, and 1.7 nmol of the enzyme in a final volume of 2.0 ml. The reaction was carried out at 25°C in an airtight tube. Throughout the experiment (Fig. 4), crotononitrile was stoichiometrically hydrolyzed, with the concomitant formation of crotonic acid and ammonia. No formation of crotonamide was noted. The results indicated

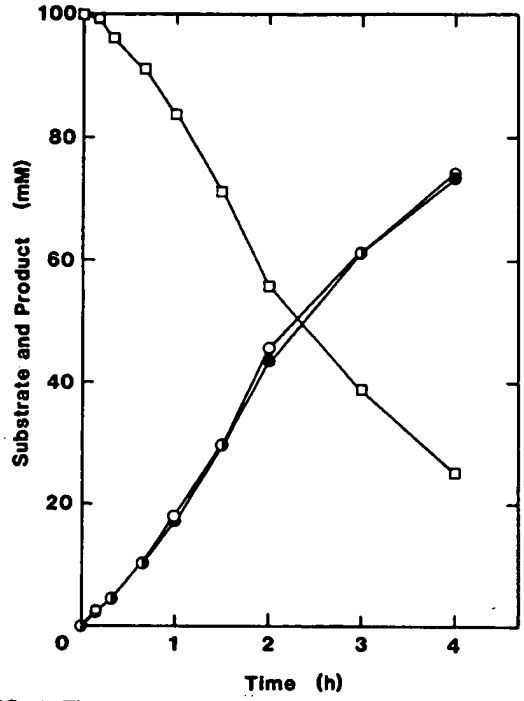

FIG. 4. Time course and stoichiometric analysis of the nitrilase reaction, using crotononitrile as the substrate. Symbols: □, crotononitrile; ●, crotonic acid; ○, ammonia. The assay conditions and method for analysis of reactant and product are described in Results and Discussion.

that crotonic acid was formed stoichiometrically with the consumption of crotononitrile. When crotonamide was added as the substrate, no formation of crotonic acid was detected. This finding suggests that crotonamide is not an intermediate in the course of nitrile hydrolysis by the enzyme. Hitherto, enzymes that catalyzed the hydrolysis of nitriles to acid and ammonia and the hydration of nitriles to amides were generically called nitrilases (9, 25). Although these enzymes belong to a group of nitrile-degrading enzymes, from these results it must be concluded that the nitrilase catalyzes only the direct hydrolysis of nitriles to acids and ammonia, without the formation of amides as intermediates, and in this respect it is completely different from nitrile hydratases.

Serological properties. The following eight *Rhodococcus* strains from the IFO, JCM, and ATCC type culture collections were examined, using crotononitrile as a substrate, for the ability to form the nitrilase: *R. erythropolis* (IFO 12539 and IFO 12682), *R. rhodochrous* (IFO 3338, JCM 2157, JCM 3202, and ATCC 21198), *R. rhoseus* (JCM 2156), and *R. rubropertinctus* (JCM 3204). Only *R. erythropolis* (IFO 12682) showed low activity (4.04×10^{-2} U and 3.32×10^{-4} U/mg).

Immunodiffusion techniques (34) were used to examine the ability of antiserum prepared against the purified nitrilase of *R. rhodochrous* K22 to cross-react with the nitrilase from *R. erythropolis* (IFO 12682). No precipitin band was formed with this enzyme. The nitrilase from *R. rhodochrous* J1 (20) and the nitrile hydratases from *R. rhodochrous* J1 (32), *P. chlororaphis* B23 (30), and *Brevibacterium* sp. strain R312 (31) also did not form precipitin bands. These results indicate that these enzymes do not share any antigenic determinants with the *R. rhodochrous* K22 enzyme.

This enzyme is recognized as having considerable commercial potential as well as being of general scientific interest. Furthermore, the development of stable activated sludge systems involving *Rhodococcus* nitrilases would represent a major advance in the treatment of the toxic wastes, i.e., nitriles, from factories, whereas the conventional activated sludge systems are highly susceptible to inactivation by the wastes. Very recently, we determined the nucleotide sequence of the nitrile hydratase gene of *R. rhodochrous* J1 (unpublished experiments). To clarify whether the *R. rhodochrous* K22 and J1 nitrilases, the *Klebsiella* nitrilase, and the *R. rhodochrous* J1 nitrile hydratase exhibit any cross-homology at the gene level, studies involving the molecular cloning of both nitrilase genes are in progress.

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